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13. SUPPLEMENTARY NOTES

14. ABSTRACT IDO1 is a tryptophan catabolizing enzyme known to support primary tumor outgrowth through immune suppression though there is little data addressing its role in metastasis, an important aspect of tumor malignancy. Tumors formed by orthotopic engraftment of the malignant 4T1 breast carcinoma cell line exhibit metastatic spread to organs similar to that seen in human breast cancer with pulmonary metastases being the primary cause of mortality in this model. To determine the role of IDO1 in breast cancer metastasis, we have utilized IDO1 knockout (IDO1-/-) mice to directly study the impact of IDO1 loss in the host. While primary tumors in IDO1-/- mice exhibited a similar growth rate to that observed in the wild-type (WT) controls, survival was significantly increased in the IDO1-/- mice. Further analysis of IDO1-/- mice showed approximately 10-fold less metastatic burden in the lungs of these mice. Serum isolated from IDO1-/- and WT controls showed similar levels of metastatic cells indicating that the reduced metastatic spread is not due to decreased tumor cell migration but rather to the reduced ability to establish tumor metastases. Evaluation of the tumor microenvironment showed that IDO1 protein and activity in WT mice directly correlates to metastatic burden. The immune cell profile in these two populations differs, leading us to conclude that IDO1 expression in normal lung tissue influences the immune response and supports the development of metastases.

15. SUBJECT TERMS
Indoleamine 2,3-dioxygenase, pulmonary metastasis, microenvironment, immune response

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INTRODUCTION:

Treatment of cancer commonly entails surgical resection followed by chemotherapy and radiotherapy, a regimen that results in variable degrees of long-term success. This is in part due to the ability of tumor cells to escape these methods of treatment and restore primary tumor growth and more importantly distant metastasis. The majority of cancer related deaths are due to the development of metastatic disease as opposed to primary tumor burden. In human breast cancer, the lungs are the primary site of metastasis followed by bone (1,2). Evidence of metastasis in breast cancer patients is considered a strong negative prognostic factor. Therefore advances in treatment to reduce metastasis will greatly improve survival in breast cancer.

The finding that there is a synergistic benefit to combining chemotherapy with the indoleamine-2,3-dioxygenase (IDO1) inhibitor 1-methyl-tryptophan (1MT) in preclinical mouse models of breast cancer (3) suggests a promising new therapeutic approach to the treatment of breast cancer. IDO1 is the rate-limiting factor in tryptophan catabolism; however, it is not involved in dietary catabolism in the liver, leading researchers to determine an alternative role for this enzyme. The seminal demonstration that 1MT could elicit MHC-restricted T cell-mediated rejection of allogeneic mouse concepti (4,5) established a role for IDO1 in mediating immune tolerance. Studies have also revealed a pathophysiological link between IDO1 and cancer, with increased levels of IDO1 activity being associated with a variety of different tumors (6,7). The therapeutic potential of targeting IDO1 in conjunction with chemotherapy has been demonstrated in the MMTV-Neu mouse model of breast cancer. The effects of 1MT were found to be greatly enhanced when given in conjunction with the commonly used chemotherapeutic agent paclitaxel (3). Depletion of either CD4+ or CD8+ T-cells in these mice abolished the benefit provided by 1MT indicating the importance of T cell immunity in the antitumor response.

The immunosuppressive function of IDO1 manifests in several manners. Collectively, IDO1 and its metabolites can directly suppress T cells (17-20) and NK cells (21) as well as enhance local Tregs (22). While there are no currently published studies, the protumorigenic capabilities of myeloid derived suppressor cells (MDSCs)(13-16) suggest that this population may also be affected by IDO1. Furthermore, IDO1 is produced in response to IFN-γ, an important cytokine modulator of inflammation. It is therefore reasonable to hypothesize that IDO1 not only regulates immune cells but may be regulated by or may regulate cytokine production in the host that results in a protumorigenic microenvironment.

To address these questions, we have characterized tumorigenesis in IDO1-/- mice to allow for the study of IDO1 in metastasis. Using an immune competent model we focused on the role of IDO1 in immune response to tumors. We have selected the highly metastatic 4T1 breast cancer model which progresses similarly to human breast cancer (10,12). Using this model, we were able to compare the metastatic sites of IDO1-/- and WT mice and evaluate the importance of IDO1 in metastatic outgrowth. Our studies in this model demonstrate that the loss of IDO1 improves survival due to reduced pulmonary metastasis and furthermore, this occurs through the reduced immunosuppressive response of the host.

BODY:

Prior to commencement of all animal related experiments, IACUC approval to conduct the proposed experiments was obtained. In our **first specific aim** we proposed to evaluate IDO activity in the lungs of 4T1 tumor-bearing mice and determine the source of its expression. 4T1 cells were orthotopically injected into the mammary fatpad of BALB/c and IDO1-/- mice. After 2 weeks, palpable tumors were observed and two perpendicular measurements were taken at weekly intervals over a six week period to evaluate primary tumor growth. Primary tumors in IDO1-/- mice exhibited a similar growth rate to that observed in the wild-type control (**Fig. 1A**). However, survival was significantly increased in the IDO1-/- mice by an average of 22 days (**Fig. 1B**). The 4T1 model is a well characterized system to replicate stage IV breast cancer due to the spontaneous metastasis to lungs, liver, lymph node and brain. The highly metastatic nature of 4T1 suggested that the difference in survival, while not related to primary tumor burden, may be a result of disproportionate metastatic burden. Therefore, lung, liver and brain were harvested along with blood and analyzed by the clonogenic assay for metastatic tumors. Metastatic colonies in the liver and brain did not exceed 10 per organ and had no statistical difference between WT and IDO1-/- mice. However, metastatic colonies in the lung showed that there was less metastatic burden in the lungs of IDO1-/- mice by approximately 10-fold (**Fig. 1C**). Serum isolated from IDO1-/- and wild-type controls showed similar levels of metastatic cells indicating that the reduced metastatic spread is not due to decreased tumor cell migration but rather to the reduced ability to establish tumor metastases (**Fig. 1C**). The observation in lung was further confirmed using both India Ink re-inflation of the lungs and microCT (**Fig. 1D**). Injection of India Ink into the lungs allows normal lung tissue to absorb the stain causing metastatic regions to be visualized as white nodules. Similarly, microCT shows normal lung as a dark region while the significantly denser metastatic regions appear white, further confirming that the improved survival of IDO1-/- mice is due to reduced pulmonary metastasis.

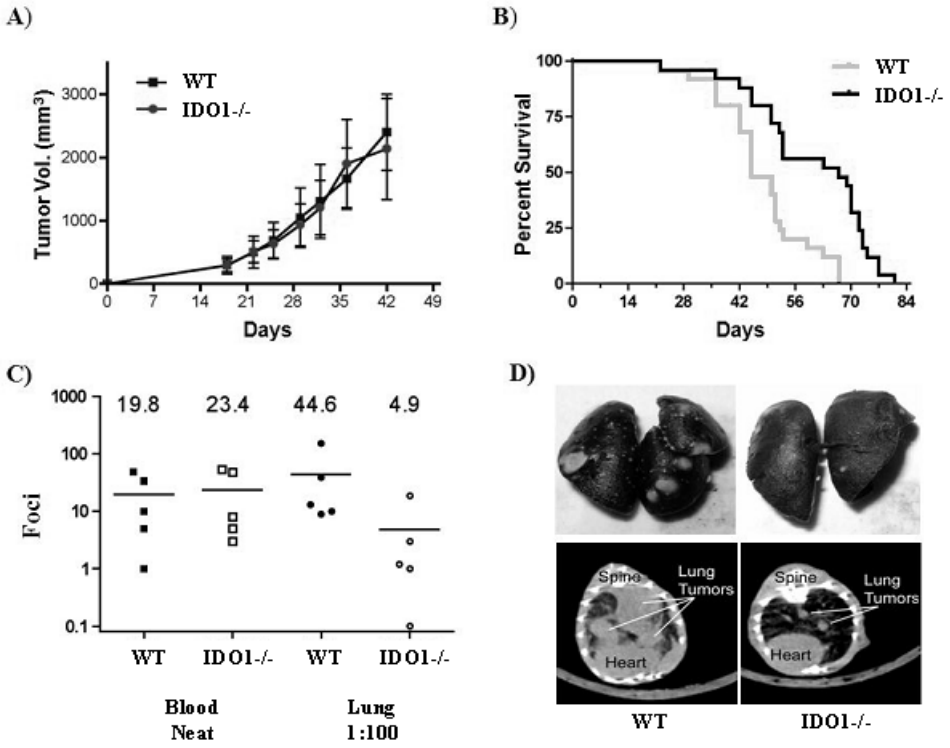

Figure 1: IDO1 is critical in pulmonary metastatic outgrowth. Mice were injected orthotopically with 4T1 cells (A-D). (A) Primary tumor burden was measured in WT and IDO1-/- mice showing no difference in growth rate. Data are representative of 3 experiments. (B) Survival studies were plotted as a percent survival over time. Significance was determined by a 2-group log-rank test (Mantel-Haenszel test). (Increased median survival = 22 days, $P < 0.0001$) (C) Number of metastatic colonies determined by clonogenic assay at 6 weeks following 4T1 engraftment for individual mice are plotted with a mean value for each group and SEM. Statistical analysis performed using the 2-tailed Mann-Whitney test. Data are pooled from two independent experiments. (D) Visualization of pulmonary metastases by India Ink (upper) and microCT (lower).

The reduction in metastasis observed in the IDO1-/- mice suggests that IDO1 is pro-metastatic. We therefore investigated the presence of IDO1 protein in the microenvironment of the lung. Time points were collected at 1 week intervals between 2-6 weeks for WT and 2-8 weeks for IDO1-/- mice. Baseline levels were obtained from non-tumor-bearing mice. In WT mice early metastases are observed at approximately 3-4 weeks. By 5 weeks there is a substantial metastatic burden, leading to a lethal burden in the 6^{th} week. By comparison, these timepoints are shifted two weeks later for IDO1-/- mice requiring IDO1-/- timepoints to be extended to 8 weeks. No data at 7 and 8 weeks is available for WT mice as they do not survive past 6 weeks. Protein IDO1 was measured by immunoprecipitating with αIDO1 and probing a Western blot with another IDO1 antibody. As expected, IDO1-/- mice do not express any IDO1 protein, however, WT mice have greater levels of IDO1 as metastatic burden increases (**Fig. 2A**). Using LC/MS/MS to measure the presence of kynurenine, a product of tryptophan catabolism by IDO1, we were able to demonstrate that the IDO1 protein in the lung microenvironment is also functionally active and that the level of kynurenine increases directly in relation to the protein (**Fig. 2B**).

This further supports the importance of IDO1 in the lungs for optimal conditions of metastatic outgrowth.

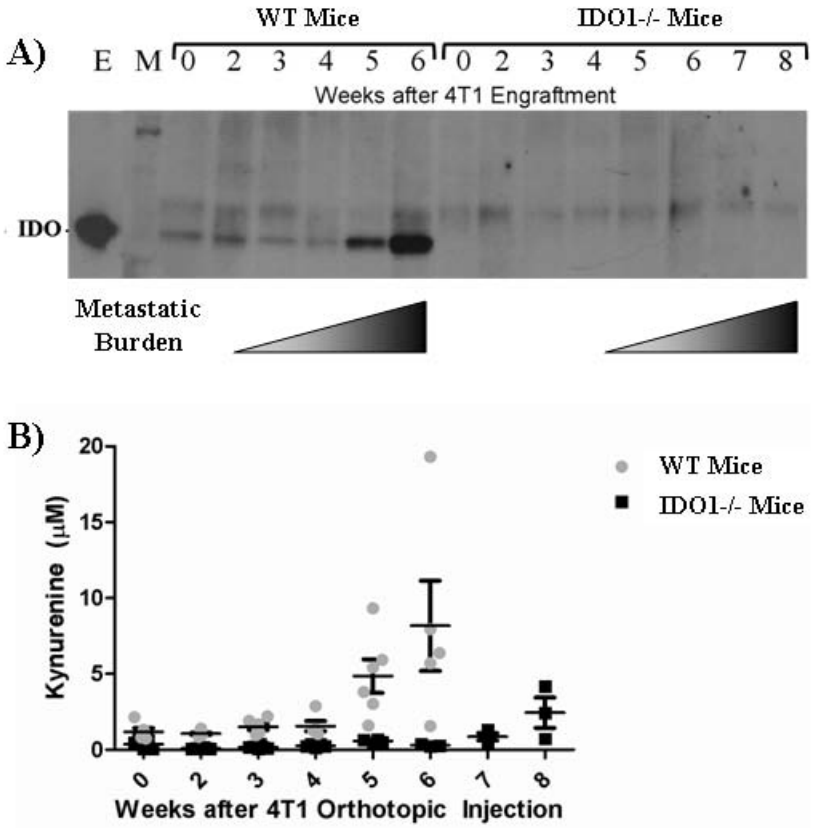

Figure 2: IDO1 increases with metastatic burden. (A) Immunoprecipitation of lung homogenate using αIDO1 was run on a Western blot and probed with anti-IDO1. (B) Kynurenine levels present in homogenized lung from WT and IDO1-/- mice were measured by LC/MS/MS.

Current roadblocks relate to the difficulty of obtaining an IDO antibody suitable for immunohistochemistry. Until recently, existing antibodies have not been used on IDO1-/- mice and therefore no publications of IDO immunohistochemistry have this control. Our lab and others have observed high levels of non-specific staining with these antibodies in IDO1-/- samples, suggesting that these antibodies are not effective for IHC purposes. Currently we are collaborating with other researchers at LIMR to develop our own antibody to IDO1. However, a recent E-publication in Prostate from the laboratory of Tomas Leanderson (23) showed negative IHC staining in IDO1-/- mice using a commercial antibody. This has provided the first example of true negative staining and we are testing their protocol on our samples.

While we continue to optimize the IHC staining of IDO1, we have been able to complete the work proposed in the other subaims of Aim 1. We are currently setting up the repeat of 4T1 tumor engraftment, to be completed as proposed, early in the second year of funding.

The **second specific aim** is designed to characterize the immune response in the lungs of 4T1 tumor-bearing mice. To determine which immune cells may be involved, an infiltrating immune cell profile was evaluated for differences between the WT and IDO1-/- 4T1 tumor-bearing mice. Lung tissue was enzymatically dissociated to form single cell suspensions for analysis by flow cytometry using the following panel of antibodies: αCD45, αCD4, αCD8, αCD3, αB220, αCD11c, αCD11b and αGr1. Lung samples were collected at 1 week intervals between 1-5 weeks following tumor engraftment. Between 2 and 3 weeks, the immunosuppressive MDSC population, identified here as CD11b+Gr1+ was greatly increased in the WT mice (**Fig. 3**). The data collected from these studies represent the percentage of positive cells from each immune cell population out of the entire CD45+ population. While this is useful to get an idea of the content, we have determined that it will be beneficial to repeat these studies and measure total cell numbers. We can interpret the current data to indicate greater numbers of MDSCs in WT mice, we cannot as cleanly evaluate the other populations. The higher MDSC production reduces the percentage of CD4+, CD8+ and B220+ cells. These reduced percentages cannot be interpreted as a lower cell number without using the total cell count. By analyzing the total number of cells of each type, we will be able to determine if there is a change in the other cell types. Interestingly, while the CD4+, CD8+ and B220+ populations are increased as a percentage in IDO1-/- mice, the CD11c+ population remains equal between WT and IDO1-/-. This suggests that there may be a difference in CD11c+ cells that is being obscured by the MDSC population while artificially enhancing the difference in CD4+, CD8+ and B220+ cells.

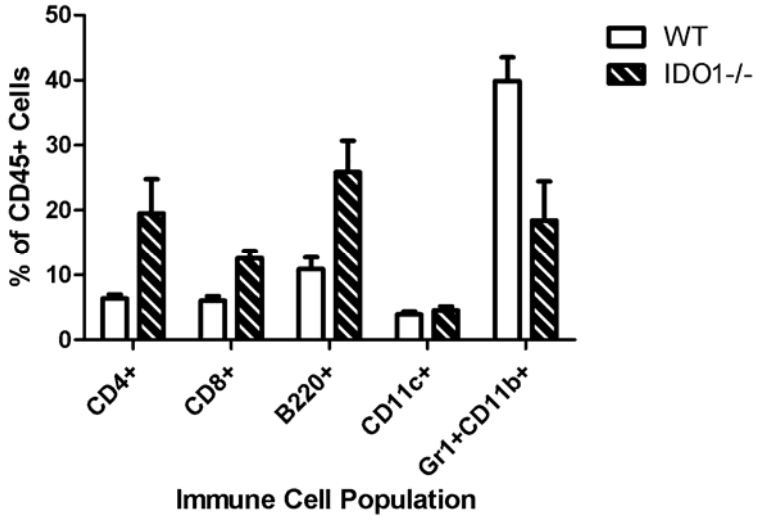

Figure 3: MDSCs elevated in WT mice. (A) Whole lung tissues from WT and IDO1-/- mice were enzymatically digested to single cell suspensions for analysis by flow cytometry. Immune cells were selected by gating on CD45+ cells. Cells were further identified for cell type by the following markers: CD3+CD4+ (helper T-cells), CD3+CD8+ (cytotoxic T-cells), CD3-B220+ (B-cells), CD11c+ (alveolar macrophages and dendritic cells) and Gr1+CD11b+ (MDSCs).

The characterization of the immune response may also be confirmed by the use of immunohistochemistry. Due to the complications with IDO1 detection, we have not been able to accomplish Specific Aim 2.c. However, we were able to begin the Bone Marrow Chimera Test Runs at the end of this month (Aim 2.e) which were originally scheduled for Year 2. To effectively determine the reconstitution of the irradiated mice, we proposed the use of C.B-17 mice. C.B-17 mice are a congenic strain on a BALB/c background that carries the immunoglobulin heavy chain allele (Igh-1b), found on C57BL6 mice. This provides a marker to determine the level of reconstitution. Our pilot run has shown that we have greater than 99% successful reconstitution in our mice. Furthermore we found that the use of C.B-17 mice in place of BALB/c, did not affect the rate of lung metastasis as determined by the number of circulating tumor cells and number of pulmonary metastases counted by the colony forming assay **(Fig. 4)**.

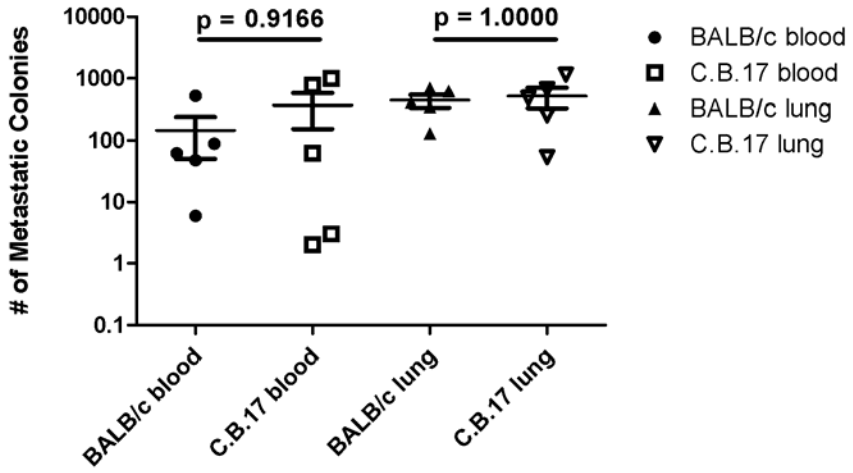

Figure 4: BALB/c mice and C.B-17 mice have equal metastatic burden. Lung tissue was homogenized and blood collected 6 weeks following orthotopic engraftment of 4T1 cells into BALB/c and C.B-17 mice. Colony forming assay was utilized to measure metastatic spread.

The second aim will be continued into Year 2 as we optimize the immunohistochemistry. We have begun the repeat analysis of infiltrating immune cells focusing on total numbers and not absolute values. The test run of the bone marrow chimera mice has shown that we can substitute C.B-17 mice for BALB/c (WT) mice in our studies allowing us to utilize this model for our experiments. We will be starting the bone marrow chimera experiments within the first six months of Year 2. During Year 2 we anticipate concluding the proposed experiments from Specific Aim 1 and the majority of Specific Aim 2. Experiments from Specific Aim 3 are currently budgeted to commence in Year 3.

KEY RESEARCH ACCOMPLISHMENTS:

- The improved survival of IDO1-/- mice is due to reduced pulmonary metastasis
- Confirmed that expression of IDO protein is present in the metastatic site of the lung in orthotopically engrafted 4T1 breast cancer model and correlates with activity as evidenced by increased kynurenine production
- Demonstrated that equal numbers of circulating tumor cells are observed in WT and IDO1-/- mice suggesting the effect of IDO loss and improved survival is due to a decreased adherence, extravasation or metastatic outgrowth
- The immunosuppressive MDSC population increases more rapidly in WT mice compared to IDO1-/-

REPORTABLE OUTCOMES:

- Invited Speaker for Keystone Symposium on Molecular and Cellular Biology of Immune Escape in Cancer, Feb 7-12, 2010, Keystone Resort, Keystone, CO

- Travel Award/Conference Assistant for Keystone Symposium on Molecular and Cellular Biology of Immune Escape in Cancer, Feb 7-12, 2010, Keystone Resort, Keystone, CO

- Poster Presenter for Joint AACR Conference on Metastasis and the Tumor Microenvironment, Sept 12-15, 2010, Philadelphia, PA

- Manuscript in prep. **Smith, C.,** Duhadaway, J., Ostrand-Rosenberg, S., Muller, A.J., Prendergast, G.C. Indoleamine 2,3-dioxygenase (IDO1) Supports Metastatic Outgrowth in the 4T1 Breast Cancer Mouse Model

- Submitted book chapter. Prendergast, G.C., Metz, R., Chang, M.Y., **Smith, C.,** Ostrand-Rosenberg, S., Muller, A.J. 2010. Indoleamine 2,3-dioxygenase and tumor-associated macrophages: possible connection in cancer-associated inflammation and immune escape?

CONCLUSION:

During the course of our studies, we have found several additional experiments that would improve upon the current data. Studies to show the importance of primary tumor on metastasis would be advantageous. This would require resection of primary tumor and/or tailvein injection of 4T1 cells. Additionally, we also propose to expand to a second model of metastatic breast cancer, E0771, in C57/BL6 mice. This would support our current experiments as well as expand the potential mouse models that can be bred onto this strain.

There are over 40,000 deaths each year in the US resulting from the metastatic spread of breast cancer. Based on data from our lab and others, an IND (investigational new drug) application for the IDO inhibitor D-1MT was approved this year and Phase I clinical trials with

D-1MT have recently commenced. Breast cancer is identified in the clinical development plan as one of the high priority disease indications for evaluation in Phase IIA studies that will be used to determine the clinical scenarios best suited for the Phase II/III clinical development of this agent. Our recently published finding that D-1MT treatment in combination with cyclophosphamide chemotherapy significantly improved survival in mice bearing highly malignant 4T1 tumors (9) suggests for the first time that this approach may be applicable to metastatic disease as well.

Using the IDO-knockout mouse strain, we have been able to genetically establish that IDO is important for supporting the development of pulmonary metastases from orthotopic 4T1 tumors. The core goal of the proposed project is to determine the underlying biological basis for this pro-metastatic effect of IDO. The data produced by these studies will elucidate what we anticipate will be a novel mechanism of action through which IDO inhibitors can enhance the antitumor immune response achieved with cyclophosphamide chemotherapy, a frontline agent for the treatment of breast cancer patients. The results of these studies could have immediate bearing on how future clinical trials with IDO inhibitory compounds are designed and may lead to the development of more effective strategies for administering IDO inhibitors for the treatment of patients with metastatic breast cancer.

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